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(57) Abstract

Herein described are therapeutic compositions that contain, as active ingredient, hyaluronic acid fragments that are characterized structurally by a chain length of from 5 to 16 monosaccharides, and by a GlcNAc residue at the non-reducing end. A particularly preferred fragment is the nonameric GlcNAc-[GlcUA-GlcNAc]4. The fragments are useful particularly to effect localized bone repair, such as for the treatment of bone fracture.

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HYALURONIC ACID FRAGMENTS AND THEIR THERAPEUTIC USE

Field of the Invention

This invention relates to hyaluronic acid fragments and to their therapeutic use, particularly for the treatment of bone.

Background to the Invention

Hyaluronic acid (HA) exists naturally, and usually in salt form, as a high viscosity glycosaminoglycan that can be extracted from such diverse sources as rooster comb, umbilical cord, vitreous humor, synovial fluid, pathologic joints, skin and group A and C hemolytic Streptococci. It is polymeric in structure and consists of alternating N-acetyl-ß-D-glucosamine (GlcNAc) and ß-D-glucuronic acid (GlcUA) residues linked at the 1,4 and 1,3 positions, respectively. Extraction from tissue provides HA in its native, high molecular weight form, generally within the size range from 50,000 to 8,000,000 daltons. In this high molecular weight, gel-like state, HA has been used therapeutically, for example as an ophthalmic surgery aid and to treat arthritic or otherwise inflamed joints (see US 5,079,236). It has also found application in the cosmetic industry, as a deeply penetrating, moisturizing agent (GB 2,099,926).

The high molecular weight form of HA has also found use as a vehicle for delivering therapeutic agents. For wound healing and tissue repair applications, for example, WO90/05522 has suggested the combination of HA and various growth factor proteins such as platelet-derived growth factor, epidermal growth factor, fibroblast growth factor, nerve growth factor and the like. US 5,095,037 suggests the use of HA as a vehicle for delivering non-steroidal anti-inflammatory agent to an arthritic site, there being some indication that HA itself may contribute to the anti-inflammatory response. WO91/04058 suggests

that HA serves its delivery role by enhancing cell permeability to the drug being delivered.

It has also been suggested that the strongly electronegative charge on the HA polymer, its chemotactic effect on various cell types and its biodegradability make it well suited for use as a vehicle for delivering bone active agents. US 5,133,755 discloses a bone implant having a porous matrix impregnated with HA, which serves as carrier for the delivery of bone active agents such as bone morphogenetic proteins. Similar bone treatment implants, comprising HA in a collagen base, have been described in WO91/18558 and WO91/17777.

Sheering of high molecular weight HA, for example by heating or mechanically, generates smaller molecular weight forms that have also found utility in the pharmaceutical and cosmetic arts. Fragments of about 25,000 daltons and greater, for instance, are described in WO92/10195 as having cell proliferation activity, and are proposed for use in aqueous gel form to treat ulcers and other damaged tissues that can benefit from increased blood flow.

Still smaller fragments of HA have been generated either by enzymatic digestion or by chemical synthesis, to study the three dimensional structure of HA. Cowman et al digested HA with each of leech hyaluronidase and testicular hyaluronidase, and then isolated fragments ranging in length from one to several disaccharide units, for analysis by circular dichroism (see Biochemistry, 1981, 20:1379), and by NMR (see Arch. Biochem. Biophys., 1984, 230(1):203). Similarly, Turner et al analyzed the dye-binding characteristics of such fragments, with a view to developing a colorimetric assay of HA fragment length (see Archives of Biochem. Biophys., 1985, 237(1):253. The interaction between HA and cartilage proteoglycans has been

studied also using HA fragments obtained by enzymatic digestion (Christner et al, J. Biol. Chem., 1979, 254(11):4624; and Hascall et al, J. Biol. Chem., 1974, 249(13):4242). Also, the interaction between hyaluronectin, HA and HA fragments has been investigated (Bertrand and Delpech, J. Neurochemistry, 1985, 45:434).

In addition to structure studies, short HA fragments in the 7-50 monosaccharide range have been examined for angiogenic activity (West et al, Science, 1985, 228:1324), and are proposed for dermal use optionally in combination with a hair growth stimulant (EP 295,092).

Summary of the Invention

A correlation has now been discovered between the length and configuration of hyaluronic acid fragments and their potency in a therapeutic context. It is therefore an object of the present invention to exploit for therapeutic purposes those hyaluronic acid fragments that exhibit relative greater potency.

More particularly, and according to one aspect of the invention, there is provided a composition comprising a physiologically acceptable carrier and, as active ingredient, a therapeutically useful amount of a hyaluronic acid fragment having a chain length in the range from 5 to 16 monosaccharides and a configuration in which a GlcNAc residue occupies the non-reducing end.

In one embodiment of the invention, such compositions are in a form suitable for application to a bone surface, and contain the hyaluronic acid fragment in an amount effective to induce bone growth, for example at a bone fracture site. In a preferred embodiment, the carrier comprises an osteoconductive matrix material for localizing the fragments and accommodating new bone growth

at the site of delivery. In an alternative embodiment, the hyaluronic acid fragments of the invention are applied to the bone-engaging surface of a bioimplant, such as a metal or ceramic prosthesis, to promote bone ingrowth.

In a further aspect of the invention, there is provided a method for inducing bone growth in a mammal including a human, comprising the step of administering a composition of the present invention to a bone surface at which bone growth is desired.

These and other objects of the present invention are now described in greater detail with reference to the accompanying drawings in which:

Brief Reference to the Drawings

Figure 1 compares activities of hyaluronic acid fragments;

Figures 2 and 3 illustrate osteogenic activity of embodiments of the invention; and

Figure 4 illustrates osteogenic activity of a preferred embodiment of the invention.

Detailed Description of the Invention and Preferred Embodiments

The invention relates to therapeutically useful hyaluronic acid (HA) fragments that are characterized structurally by a chain length in the range from 5 to 16 and by a configuration in which a GlcNAc residue is at the non-reducing end. It has been found that HA fragments having these features exhibit therapeutic potency that is surprisingly far greater than other HA fragments having identical configuration but different length, and greater

than HA fragments having identical length but different configuration, particularly those in which GlcUA occupies the non-reducing end.

The term "reducing end" refers to the terminal monosaccharide having a C-1 hydroxyl group that is not involved in glycosidic linkage. The term "chain length" refers simply to the number of linked monosaccharides resident in the fragment. The term "configuration" refers to the identity of those monosaccharides, particularly with respect to the terminal monosaccharides, i.e. the reducing and non-reducing ends of the fragment. An HA fragment species having a configuration in which the non-reducing end is GlcNAc is a species having one of two possible structures, i.e. [GlcNAc-GlcUA]n and GlcNAc-[GlcUA-GlcNAc]n.

In a preferred aspect of the invention, the hyaluronic acid fragment has a structure of the formula:

GlcNAc - [GlcUA - GlcNAc]n

wherein n is an integer selected from 2, 3, 4, 5, 6 and 7.

The invention also embraces salts of the present HA fragments, particularly the inorganic salts including sodium, potassium and calcium.

The above formula uses conventional notation to represent the monosaccharides native to hyaluronic acid, i.e. the term GlcNAc refers to N-acetyl-ß-D-glucosamine, and the term GlcUA refers to ß-D-glucuronic acid. These monosaccharides are linked in the manner intrinsic to hyaluronic acid, that is, by ß(1-4) linkage between GlcNAc-GlcUA and by ß(1-3) linkage between GlcUA-GlcNAc.

Of the HA fragments embraced by the formula, the species presently preferred, for their greater relative potency as bone therapeutics, are those in which 'n' is selected from 2, 3, 4 and 5. Particularly preferred species are the pentamer in which 'n' is 2, the hepatmer, in which 'n' is 3 and, more preferably, the nonamer, in which 'n' is 4.

For therapeutic use, the present HA fragments can be obtained by various means, such as by *de novo* chemical synthesis from monosaccharide units, and by enzymatic digestion of high molecular weight HA, which can itself be obtained either from known sources using established techniques or directly from numerous commercial sources. Conveniently, and according to one embodiment of the invention, the fragments are generated following controlled enzymatic digestion and/or chemical cleavage of the higher molecular weight form of HA, and then isolated and purified using otherwise established biochemical techniques.

The HA fragments can be generated enzymatically by incubation with commercially available enzymes capable of cleaving one or other of the specific glycosidic linkages within the HA chain, to attain the fragment chain length and configuration desired. HA fragments having a GlcNAc residue at the non-reducing end and a GlcUA residue at the reducing end can be produced simply by an enzymatic process in which a higher molecular weight HA is incubated with a hyaluronidase of the class E.C. 3.2.1.36, such as the commercially available leech hyaluronidase, thus yielding fragments of the structure $[GlcNAc\text{-}GlcUA]_{3\text{-}8}$, among others. To ensure that desired chain length is achieved, reaction progress can be halted at the appropriate stage simply by heating to denature the enzyme, and the resulting fragments can then be isolated by size selection using techniques that are described in greater detail below.

According to one embodiment of the invention, generation of preferred HA fragments, of the formula GlcNAc-[GlcUA-GlcNAc]$_{2-7}$, can be achieved by a four step process that entails (1) digestion of the high molecular HA substrate with a hyaluronidase of the class E.C. 3.2.1.35 to liberate a series of precursor fragments differing in chain length and having the configuration [GlcUA - GlcNAc]n; (2) separating the precursor fragments according to size and isolating those having a chain length of 6 to 16 (n=3-8); (3) treating the isolated fragments with an enzyme of the class E.C. 3.2.1.31 to remove GlcUA selectively from the non-reducing end, thus yielding a desired fragment conforming to the formula GlcNAc - [GlcUA - GlcNAc]$_{2-7}$ and then (4) purifying the desired fragment such as by chromatography, for example using HPLC techniques.

More particularly, to generate initially the precursor fragments [GlcUA - GlcNAc]n, high molecular weight HA in desirably purified form and obtained from a commercial source for instance, is preferably treated with an endoglycosidase of the E.C. 3.2.1.35 class that is specific for HA, such as the commercially available testicular hyaluronidase, of bovine origin for instance. Enzymes of this class have the desirable property of acting selectively on HA, to the exclusion of other sulfated glycosaminoglycans that may contaminate the substrate preparation. Because digestion with this enzyme will yield mostly tetra- and hexasaccharide end products if left to completion, progress of the reaction is necessarily controlled when fragments having a longer chain length are desired. Such control can be achieved simply by heating to inactivate the enzyme at a reaction stage determined empirically to be optimal for producing fragment precursors having the target length.

In the next step of fragment generation, fragment precursors in the reaction mixture are physically separated

to isolate those of target length. This separation can be achieved on the basis of size alone for example by gel exclusion chromatography; on the basis of charge alone for example by capillary electrophoresis; on the basis of charge-to-mass ratio for example by gel electrophoresis; or on the basis of hydrophobicity for example by reversed phase HPLC on amino-bonded silica. Methods for detecting the precursor fragments either as the reaction progresses or after their recovery include UV spectrophotometry or colorimetric assays specific for D-glucuronic acid and N-acetyl-D-glucosamine residues. The amount, purity and length of each fragment precursor so isolated can be determined without ambiguity using analytical techniques well known in the oligosaccharide art.

Once collected, the isolated precursor fragments of formula $[GlcUA - GlcNAc]_{3-8}$ are trimmed to remove the non-reducing GlcUA residues, using an exoglycosidase specific for HA (E.C.3.2.1.31), particularly ß-glucuronidase extracted for instance from rat prepubital gland or from bovine liver. The reaction can proceed to completion, and the resulting digestion mix treated such as by chromatography to isolate and purify fragments having the desired structure, $GlcNAc-[GlcUA-GlcNAc]_{2-7}$.

If desired, any of the isolated HA fragments can be desalted on ionic exchange columns and subsequently converted, if desired, to a distinct salt after titration with suitable base.

For use therapeutically, the present HA fragment composition desirably incorporates a pharmaceutically acceptable form of the selected fragment, lacking contaminants such as nucleic acids, amino acids, pyrogens and other or higher molecular weight oligosaccharides including other HA fragments in amounts that are significant from a regulatory viewpoint. In a preferred

embodiment of the invention, the pharmaceutical composition contains, as active ingredient and in a therapeutically useful amount, only or substantially only one fragment species. Compositions having "substantially" only one fragment species may contain other HA fragments or impurities in small, incidental amounts. In an alternative embodiment of the invention, the pharmaceutical composition may contain, as active ingredient and in a therapeutically useful amount, a mixture of two or more fragment species, to the substantial or complete exclusion of other HA fragment types such as those having the configurations GlcUA - [GlcNAc - GlcUA]n and [GlcUA - GlcNAc]n.

Compositions containing the present HA fragments are prepared by combining a therapeutically useful amount of at least one species, and preferably only one species, of the present HA fragments with a physiologically acceptable carrier.

The term "therapeutically useful amount" is used herein to denote an amount of the HA fragment that in unit dosage form or as part of a dosage regimen is capable of eliciting a therapeutically desirable response following administration thereof. The term "physiologically acceptable carrier" is intended to embrace vehicles used to deliver active ingredients by such industries as pharmaceuticals, biomaterials, veterinary and cosmetics, and otherwise refers to a carrier that is physiologically tolerated by the recipient.

Selection of the carrier and of the amount of HA fragment for incorporation in the composition will of course depend on many factors, particularly the disease or condition to be treated. For dermal application, such as for the treatment of burns, ulcers and other skin conditions that would benefit from increased blood flow and from enhancement of cell adhesion, the fragments may be

formulated for topical administration as gels and hydrogels, lotions, ointments, creams and the like, optionally in the form of salves, bandages, poultices and including the more sophisticated slow-release devices recently developed.

In a preferred embodiment of the invention, the HA fragment composition is formulated and used for the treatment of bone, such as for the repair of fractured bone or in the repair/remodelling of bone including skeletal and alveolar bone. As is demonstrated in the examples herein, it has been found that fragments of the present type, and particularly the heptamer and nonamer, are remarkably far more potent as bone repair therapeutics than structurally related fragments. The present composition is useful particularly when delivered to a bone surface at which accelerated bone formation is desired. Preferably, the composition is accordingly formulated in a manner appropriate to deliver therapeutically useful amounts of the fragment to a bone surface to be treated.

For use in treating bone, the compositions are prepared by combining a therapeutically effective amount i.e a bone forming amount, of the HA fragment with a physiologically acceptable delivery vehicle. Delivery vehicles suitable for use with the HA fragments can be of a type conventionally employed in delivering to bone surfaces other bone therapeutic agents, such as the various species of bone morphogenetic protein. It is to be appreciated, in this regard, that the HA fragments are relatively far more stable to acid and moderate heat treatment, and thus can be combined with a selected vehicle under chemical and physical conditions that are far more harsh than is otherwise possible with protein-based osteogenic agents.

In a preferred embodiment of the invention, the

delivery vehicle includes as one component a biocompatible material, also referred to herein as an "osteoconductive matrix", which serves to localize the fragment(s) at the bone surface on which they are applied, and desirably also serves as a substratum that accommodates the growth of new bone at the site of application. As used herein, the term "biocompatible" refers to substances having no significantly adverse effect e.g. toxic, inflammatory or immunological effect, on bone or surrounding tissue while resident at the bone surface. Suitable for use as matrix are those substances, or combinations thereof, that when combined with the present HA fragments elicit a positive response in the rat bone growth assay herein described.

Matrix materials suitable for use in delivering the osteogenic HA fragments include bone-derived materials, such as osteogenically "deactivated" bone extract that results from demineralization of crushed mammalian bone. Most suitably, the bone-compatible matrix consists of chemically defined material, to allow for greater control during dosage formulation. In this regard, suitable matrix materials include bone-produced substances such as hydroxylapatite, and collagen. Also useful as matrix materials are calcium sulphate, tricalcium phosphate, polylactic acids including polylactide, polyglycollic acid, polyglactin and polyglactic acid, polyanhydrides, and polymethylmethacrylate. Such materials can be used alone or in any useful combination. For example, hydroxylapatite can serve as a matrix material both on its own and in combination with collagen.

In addition to matrix material, the delivery vehicle may contain a physiologically acceptable carrier or diluent. The use of such carriers and diluents is desirable particularly to prepare formulations to be delivered by injection. For example, injectable formulations of the composition can be prepared by mixing

an effective amount of HA fragment with a delivery vehicle consisting of a physiologically buffered saline solution and a selected matrix material, such as hydroxylapatite. Similarly, carriers such as cements, pastes and gels, can be employed to generate solid or semi-solid compositions that can be applied, after shaping to accommodate an implant site if desired, directly at the desired site of administration.

Alternative forms of the osteogenic composition may also be prepared that are suitable for delivering the osteogenic factor by modes other than injection. For example, porous ceramic compositions may be formulated using sintered tricalcium phosphate or coralline hydroxylapatite as matrix material, for direct application to bone either by implantation or by layering. This provides a slow release form of composition useful to repair bone defects. Reference may be made to US 4,596,574 for guidance on preparing such compositions.

The composition will be useful to repair or reconstruct various bone defects such as those arising from disease, from damage induced by trauma or arising congenitally. The compositions will also be useful in bone grafting. The particular mode of administration will depend on the nature of the bone defect to be treated. In those circumstances when bone to be treated is exposed, for example during bone grafting by surgical intervention, the composition may be layered directly on the surface by application as a cement, gel or paste or may be deposited in the form of an implant.

The composition will also be useful as a sealant or filler to accelerate the repair of various osseous defects caused by disease or trauma and which necessitate the bridging, reconstruction, recontouring or augmentation of hard tissues. It can also be used as a bone substitute

to allografts, or as an agent to coat synthetic prostheses that are relevant to the practice of orthopaedic, plastic, maxillofacial, orthognathic and dental surgery, in order to facilitate the attachment, bonding, anchorage or integration of the latter to living tissues. The stability of HA fragment incorporated in the composition offers advantages relative to protein therapeutics in the design and engineering of osteotropic surfaces and layers, including those that utilize hydroxylapatites as composite biomaterials, and which are in use for artificial implants.

In one specific embodiment of the invention, the composition is used to repair bone fractures. For this purpose, the composition can be in the form of an aqueous buffered suspension containing a suitable dose of the purified fragments in combination with a selected matrix material, such as hydroxylapatite. The composition is delivered by injection to the site at which the fracture has occurred. For fractures of a medically serious nature such as non-union fractures, surgical intervention will permit application of the composition directly to the interface between bone surfaces.

The particular dosage regimen for a given bone treatment application will ultimately be determined by the attending physician, and will take into consideration such factors as the site and severity of bone damage. Defined dosage ranges can be determined in properly designed clinical trials with patient progress being tracked periodically by x-ray monitoring or if necessary by surgical exploration. Preliminary guidance useful to determine dosage sizes appropriate for human patients can be taken from the results herein presented for the rat bone growth assay. It will be noted, for example, that one-time injection of the fragment GlcNAc-[GlcUA-GlcNAc]$_4$ at a dose of 10 nanomoles caused a significant increase (up to 10%) in rat tibia-fibula weight. Doses found to be optimal for

therapy in the context of this assay are in the range from 5 nanomoles HA fragment/mg of hydroxylapatite matrix to 15 nanomoles/mg. Significantly, the increased bone mass is sustained over a period of about one month from single injection, thus it is unlikely that a daily dosage regimen is essential in the bone healing/repair therapy.

It will be appreciated that the present fragments will also have veterinary applications. Particularly, domestic animals and thoroughbred horses in addition to humans are suitable recipients of such treatment. When used for veterinary purposes, it will be appreciated that the purity of the HA fragments can be less of a concern than for human applications.

Example 1 - Generation of GlcNAc - [GlcUA - GlcNAc]$_{2-7}$

To generate these fragments, HA was first digested with testicular hyaluronidase to yield precursor fragments of the type [GlcUA-GlcNAc]n, from which the non-reducing GlcUA residues were then removed to obtain the title fragments, by the action of ß-glucuronidase.

Particularly, 50 mg of rooster comb HA (Na salt from Sigma) were dissolved with mild boiling in 10 ml of a buffered solution containing 100 mM sodium acetate, 150 mM NaCl and 1 mM disodium EDTA and adjusted to pH 5.0. To this solution were added gelatin to a final concentration of 0.1%, and testicular hyaluronidase (Sigma H-3884; bovine testes origin) also freshly dissolved in the same buffer, to begin the digestion, such that final concentrations were about 5 mg/ml (HA) and 30 to 50 Units/ml, respectively. The incubation lasted for about 3 hours, then the reaction mixture was boiled for 3 mins to halt the reaction and flocculate the proteins. After a brief centrifugation (12,000g for 15 mins) the supernatant was collected and lyophilized. The lyophilized material can be kept in the

cold for few weeks, or immediately processed for chromatographic separation.

To separate these precursor fragments by chain length, the lyophilized digest was first dissolved in about 2.5 ml of a buffered solution containing 0.25N acetic acid and 0.28M pyridine (pH=5.1), filtered through a 0.45 μm membrane and applied to a gel filtration column (2.5 cm x 120 cm) filled with about 500 ml of Biogel P30 (fine grade, Biorad). Elution proceeded at room temperature and at a flow rate of 5-6 ml/hr, until about 300 fractions of 2.5 ml were collected. After a single lyophilization, each fraction was reconstituted in 2.5 ml of water. The carbazole assay of Bitter and Muir (Analytical Biochemistry, 1962, 4:330) was used to determine the Glucuronic acid content on small aliquots, D-glucuronolactone (Sigma G-8875) serving as a calibration standard. Independent calibration of the column provided values for the void volume (Vo= 175 ml) and total volume (Vt= 545 ml; elution volume of D-Glucuronic acid, mw=194). A clear separation of peaks, designated I to about XII by increasing order of partition coefficients (Kav), was obtained such that fractions corresponding to the most central part of these peaks were pooled, lyophilized and submitted to several cycles of reconstitution in water/lyophilization/rotary evaporation to eliminate as much pyridinium acetate as possible. Each peak was eventually dissolved in water at a concentration of about 1 μg/uL of GlcUA, the exact amount being verified after repeating the carbazole assay. On average, less than 1mg GlcUA equivalent of each peak was obtained from the digestion of 50 mg of HA.

All the precursor fragments separated from hyaluronidase digests belonged to the family [GlcUA-GlcNAc]n, where n is from 2 to 11. This was established by recurrence, once it was demonstrated that n=2 coincided

with peak I. The evidence included an average Kav value of 0.81, a fairly close agreement between the theoretical mass and the one deduced experimentally from mass spectrum analysis, and a reducing end GlcNAc : GlcUA molar ratio of 0.448 ± 002 (0.50 in theory) as determined by the carbazole and the Reissig et al. colorimetric assays. This assignment was corroborated by the fact that peak II, adjacent to peak I, had a Kav of 0.71 a molar ratio of 0.333 (theory 0.333), and a mass spectrum corresponding to n=3, while peaks IV, VII and VIII had mass spectra compatible with that of n=5, n=8 and n=9, respectively.

The purity of these fragments was estimated by using electrophoresis in 10% polyacrylamide gels as described by Min and Cowman in Analytical Biochemistry, 1986, 155:275. Virtually single bands were detected after staining with Alcian blue alone. These bands formed a characteristic ladder, and the electrophoretic mobility, relative to that of the bromophenol blue tracking dye, increased with fragment size. The relationship between the logarithm of molecular weight and Rf was linear in the range from n=5 to n=10, and n=8 was characterized by a Rf=1.00. Even after silver nitrate enhancement of Alcian blue staining and loads of up to 4 μg of GlcUA equivalent per lane, neither n=3 nor n=2 were detectable.

The fragments obtained as just described were exploited as precursors from which the desired fragments, having GlcNAc at the non-reducing end, were obtained by glucuronidase digestion. More particularly, between 300 and 600μg of the individual fragments $[GlcUA\text{-}GlcNAc]_{3\text{-}10}$ were first dried by rotary evaporation. Four hundred μL of 0.1M sodium acetate buffer (pH=4.5) were added, followed by 100 μL (50 Units) of ß-D-Glucuronidase (bovine liver origin; Sigma G-0501). Tubes were incubated at 37°C for 4hr, and the reaction was stopped by boiling for 3 min. The supernatants resulting from high-speed centrifugation were

collected, evaporated to dryness and kept in the cold. Fractionation on Biogel P30 columns as described above resulted in the identification of a major and minor peak, the latter coinciding with the elution position of free GlcUA, one of the products anticipated from the trimming action of ß-D-glucuronidase. The former peak represented the desired HA fragment. Final recovery yields varied between 20 and 100% for the predicted free GlcUA, and between 80 and 100% for the desired fragments.

The electrophoretic mobility of HA fragments having a chain length of 9 to 19 was only slightly faster than that of their corresponding precursors of chain length 10 to 20, in agreement with the fact that whereas their mass is on the average 7% lower, their mass-to-charge ratio is on the average 9% higher and influences mobility in the opposite direction. Single bands were detected in all cases except those having a chain length of 5 and 7, which are too small to retain the Alcian blue dye. Mass spectra analyses confirmed the predicted structure of the fragments.

In addition to the desirably configured HA fragments prepared as described in example 1, there were also prepared for bioactivity assessment the differently configured fragments bearing a GlcUA residues at the non-reducing end, i.e., fragments having the configurations [GlcUA-GlcNAc]n and GlcUA-[GlcNAc-GlcUA]n.

For fragments having the configuration [GlcUA - GlcNAc]n, the procedure described in example 1 was followed, except that the initial enzymatic reaction was conducted in the presence of about 200 Units/mL of testicular hyaluronidase (from Calbiochem, San Diego, CA) and for about 20 hours at 37°C.

Fragments of the configuration

GlcUA-[GlcNAc-GlcUA]$_n$ were derived by chemically trimming the reducing end of the [GlcUA-GlcNAc] fragments also obtained as described in example 1. Particularly, solutions of the [GlcUA-GlcNAc]n fragments having a chain length in the range from 10 to 20 and containing between 200 and 500 μg GlcUA equivalents were first dried by rotary evaporation, and then reconstituted into 0.4 mL of a freshly prepared solution of Na_2CO_3 (0.15M). Tubes were incubated at 37°C for 4hr, after which 0.4 mL of the pyridinium-acetate buffer (pH=5.1) was added to stop the reaction. They were stored in the cold until chromatographic separation using Biogel P30 filtration as described in example 1.

The major peak was pooled and submitted to several cycles of lyophilization and reconstitution in water. The nearly complete conversion was indicated by the presence of only minute amounts of GlcUA-containing material eluting ahead of the main peak, and which would have corresponded to the uncleaved parental fragment. Based on GlcUA equivalents, the desired products were finally recovered with an average yield of 50% .

Example 2 - Formulation and Bioactivity Assessment

The rat bone growth assay described in U.S. patent 5,169,837, incorporated herein by reference, was used to compare the osteogenic potential of the different HA fragments prepared as described in example 1. Young Wistar rats (6-8 weeks of age, 150 ± 20 g in weight) received implants of the following composition, suspended in a total volume of 0.1 mL, delivered by injection to the upper part of the tibia, in its immediate proximity and without penetrating the periosteum layer:

a. HA fragment : from 1 to 20 nanomoles
b. Hydroxylapatite matrix : 1 mg original slurry weight
c. 0.85% NaCL in water : 100 μL

The implants were prepared by mixing an aqueous solution of HA fragments together with a 10 mg/mL stock aqueous suspension of hydroxylapatite (calcium phosphate hydroxide particles in the form of a slurry in 1 mM sodium phosphate; from Sigma, H-025L, Lot # 70H8090) in the proportion 1 to 6 (v/v), followed by immediate freezing and lyophilization. Lyophilized samples were not stored for more than a week at -20°C. For administration, they were reconstituted with an appropriate volume of pure water at room temperature.

The same implant was administered to a group of at least three rats. Large variation within the group was properly attributed to errors attached to the delicate mode of implantation rather than to variations in animal responses, and experiments were repeated accordingly. A minimum of four doses were tested for any given HA fragment, covering the range from 1 to about 20 nanomoles. The percentage increase in bone mass evoked 7 days after administering the implant was calculated after measuring the total tibia/fibula dry weight of the treated versus contralaterally untreated limb in the same animal. The difference in tibia dry weight between untreated limbs of the same individual never exceeded 1%, and averaged about 0.5%.

Results of the bioassay, illustrated in Figure 1, revealed that fragments having a chain length in the range from 4 to 22 and having a GlcUA residue at the non-reducing end were inactive. Surprisingly, however, HA fragments having a GlcNAc residue at the non-reducing end demonstrated remarkable bioactivity at the same dose.

A more detailed study of the active GlcNAc-[GlcUA-GlcNAc]n family of HA fragments revealed that the osteogenic activity is related to fragment chain length. The nonamer (n=4) elicits the greatest response (an average

10 % increase in bone mass) as shown in Figure 2, and also displays the lowest half-maximal dose, at 5 nanomoles per implant (Figure 3). A characteristically sigmoidal dose-response curve is represented for the nonamer in Figure 4. The osteogenic activity declines progressively for fragments shorter and longer than the nonamer, and becomes undiscernible beyond a chain length of 15 monosaccharides (n=7). The effect induced by the presently preferred fragments is manifest principally as a visible increase in bone girth at the site of injection, as opposed to increased bone length.

As an alternative to the injectable composition used in the bioassay, there can be prepared a composition suitable for implantation into an osseous defect that has been created naturally or after surgical intervention, and which requires a reconstruction phase. For this purpose, an osteogenic implant or onlay is produced by adding dropwise and over a period of 10 min, an aqueous solution of HA fragments (300 µL volume, containing up to 300 µg) onto a block of porous hydroxylapatite replicated from Porite corals (Interpore 200 from Interpore International, Irvine, CA) that has been designed to fit the dimensions of the defect, for instance a 20mm x 16mm x 1.5mm rectangle. Once the solution has infiltrated the pores of the hydroxylapatite ceramic as uniformly as possible, the impregnated device is submitted to snap-freezing in an absolute alcohol/dry ice bath, and lyophilized, to cause adhesion of the HA fragments onto and within the matrix. The composite material thus prepared can be stored at -70°C before being inserted surgically, using for example the technique described by Miller et al in Plastic and Reconstructive Surgery, 1991, 87(1):87), into the bone defect.

WE CLAIM:

1. A composition comprising a physiologically acceptable carrier and, as active ingredient, a therapeutically useful amount of a hyaluronic acid fragment having a chain length in the range from 5 to 16 and a configuration in which a GlcNAc residue is at the non-reducing end.

2. A composition according to claim 1, wherein said fragment has a chain length in the range from 5 to 13.

3. A composition according to claim 2, wherein said fragment has a chain length in the range from 7 to 11.

4. A composition according to any one of claims 1-3, wherein the active ingredient consists of a single species of said hyaluronic acid fragment.

5. A composition comprising a physiologically acceptable carrier and, as active ingredient, a therapeutically useful amount of a hyaluronic acid fragment conforming to the formula:

GlcNAc -[GlcUA-GlcNAc]n

wherein n is selected from 2, 3, 4, 5, 6 and 7.

6. A composition according to claim 5, wherein n is selected from 3, 4, 5 and 6.

7. A composition according to claim 5, wherein n is selected from 3 and 4.

8. A composition according to any one of claims 5-7, in which the active ingredient consists of a single hyaluronic acid fragment species.

9. A composition useful to treat bone, the composition comprising, as active ingredient and in an amount effective to induce bone formation, the hyaluronic acid fragment GlcNAc - [GlcUA - GlcNAc]$_4$; and a physiologically tolerable vehicle therefor.

10. A composition according to any preceding claim, further comprising an osteoconductive matrix material.

11. A composition according to claim 10, wherein said osteoconductive matrix material comprises hydroxylapatite.

12. A method for treating a mammal to induce bone formation, comprising the step of administering to a bone site at which bone formation is desired a composition as defined in any preceding claim.

13. The method according to claim 12, wherein said composition is administered to a bone fracture site.

14. A bioimplant having applied on a bone-engaging surface thereof, a composition as defined in any one of claims 1-11.

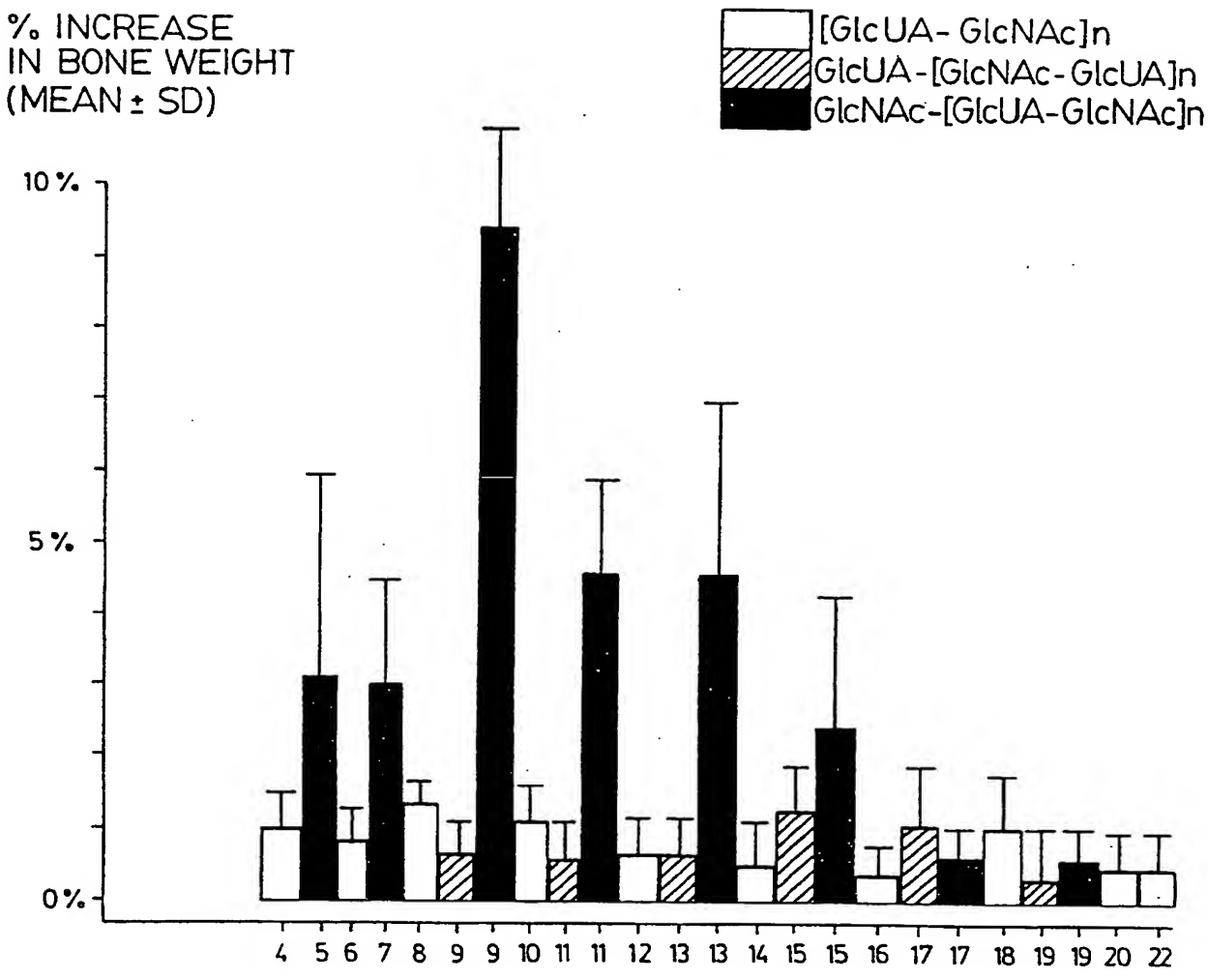

FIG. 1

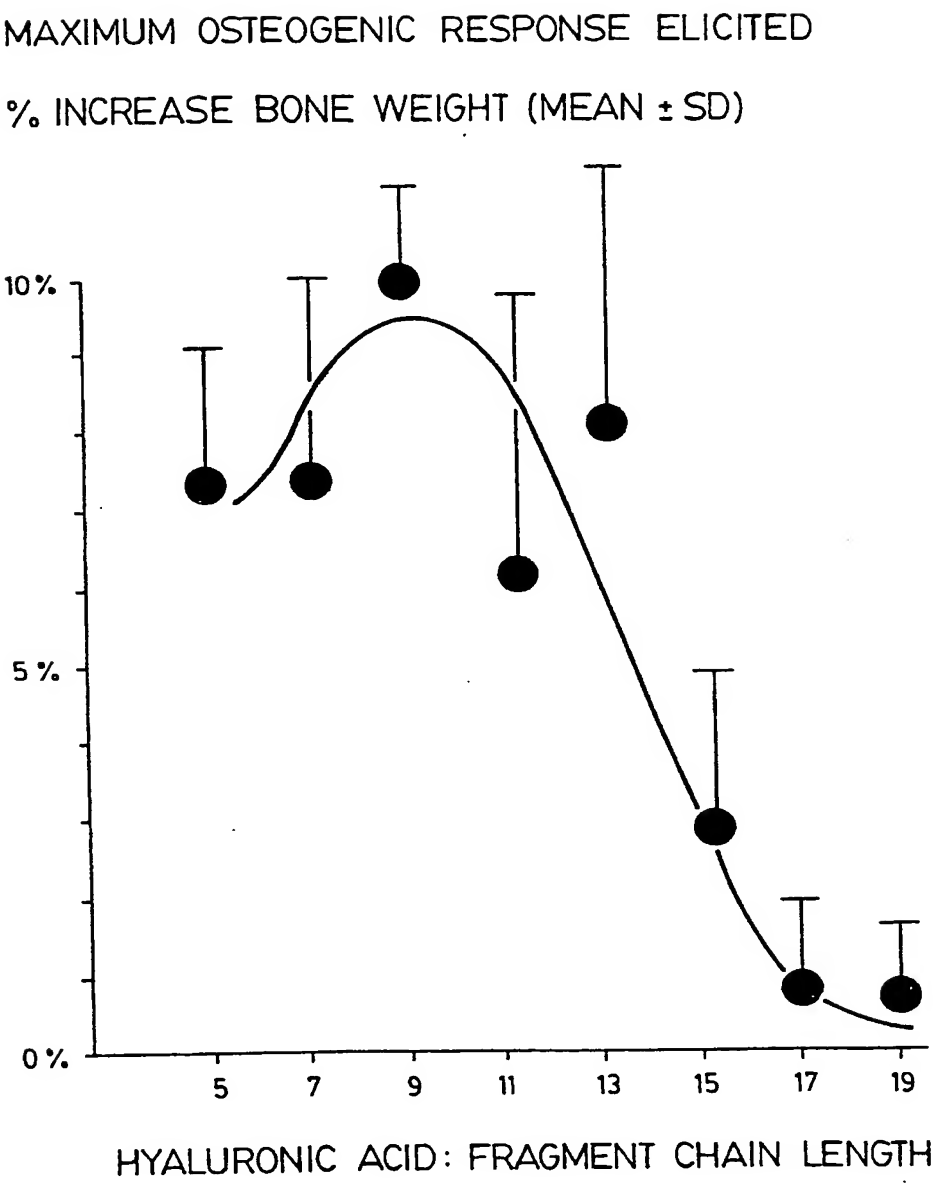
MAXIMUM OSTEOGENIC RESPONSE ELICITED
% INCREASE BONE WEIGHT (MEAN ± SD)
10 %
5 %
0 %
5
7
9
11
13
15
17
19
HYALURONIC ACID: FRAGMENT CHAIN LENGTH

FIG. 2

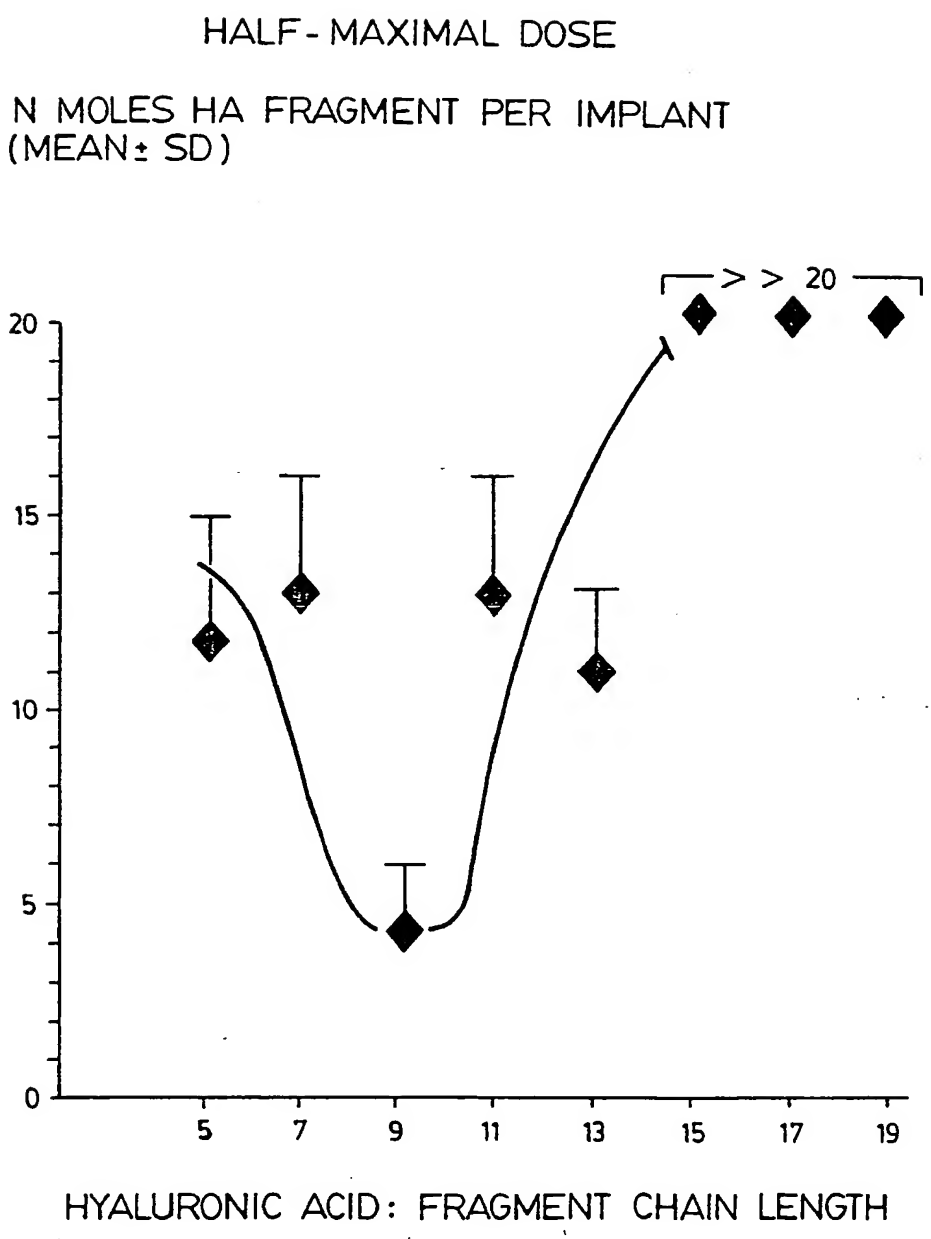

FIG. 3

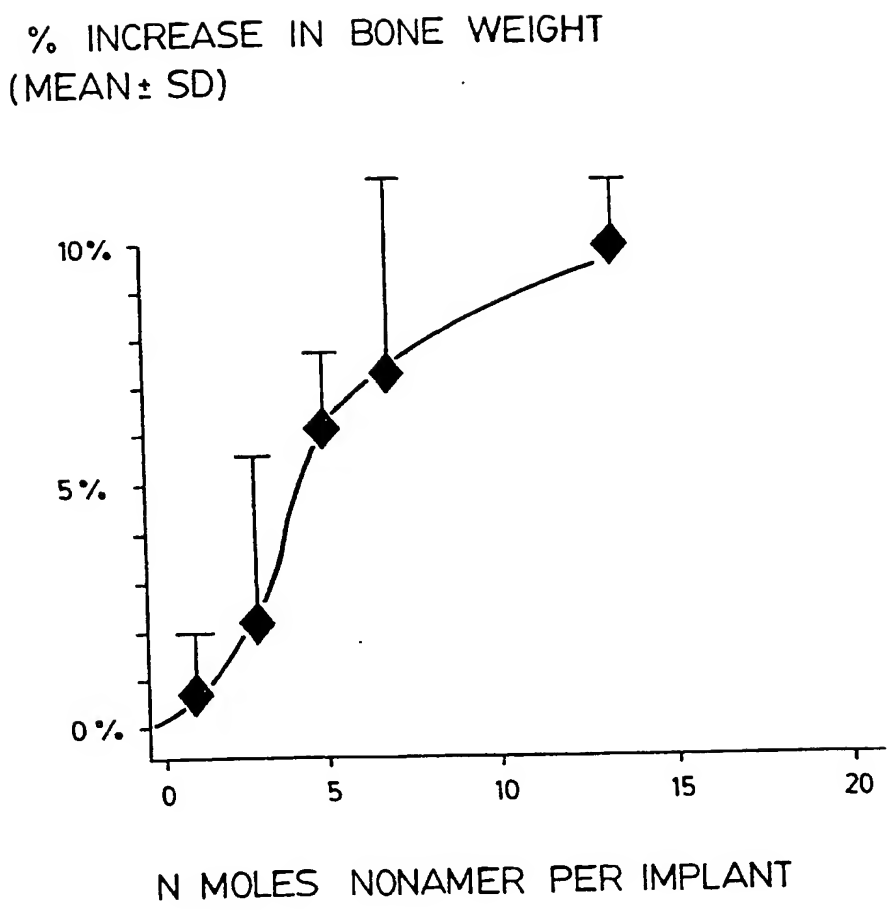
% INCREASE IN BONE WEIGHT
(MEAN ± SD)
10%
5%
0%
0
5
10
15
20
N MOLES NONAMER PER IMPLANT

FIG. 4

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/CA 94/00352

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/73

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,93 20827 (CALLEGARO ET AL) 28 October 1993 see the whole document ---	1-14
X	SCIENCE, vol.228, no.4705, 14 June 1985 pages 1324 - 1326 WEST, D.C. ET AL 'ANGIOGENESIS INDUCED BY DEGRADATION PRODUCTS OF HYALURONIC ACID' cited in the application see the whole document ---	1-9
A	EP,A,0 295 092 (UNILEVER PLC) 14 December 1988 cited in the application see the whole document --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
28 September 1994	18. 10. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Mair, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/CA 94/00352

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 10195 (SKANDIGEN AB) 25 June 1992 cited in the application see the whole document especially page 1, line 10	1-14
A	DATABASE WPI Week 9302, Derwent Publications Ltd., London, GB; AN 93-010422 & EP,A,0 522 569 (US SURGICAL CORP) 13 January 1993 see abstract	1-14

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA94/00352

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
REMARK: Although claims 12 and 13 are directed towards a method of treatment of the human/ animal body the search has been carried out and based on the alleged effects of the compositions.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intr onal Application No
PCT/CA 94/00352

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9320827	28-10-93	NONE		
EP-A-0295092	14-12-88	AU-B-	613920	15-08-91
		AU-A-	1745988	15-12-88
		DE-A-	3874813	29-10-92
		JP-A-	1013008	17-01-89
WO-A-9210195	25-06-92	AU-B-	649092	12-05-94
		AU-A-	9040991	08-07-92
		EP-A-	0560845	22-09-93
		JP-T-	6503319	14-04-94
		SE-A-	9003887	07-06-92